JC17 Rec'd PCT/PTO 30 MAY 2001

FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 19904-013 NATL
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 09/856940
INTERNATIONAL APPLICATION NO. **PCT/FR99/02939**	INTERNATIONAL FILING DATE **November 26, 1999 (26/11/99)**	PRIORITY DATE CLAIMED **November 30, 1998 (30/11/98)**

TITLE OF INVENTION
Pharmaceutical Composition Comprising An Anti-Cancer Agent And At Least A Peptide

APPLICANT(S) FOR DO/EO/US
TEMSAMANI, Jamal; KACZOREK, Michell

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☒ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☒ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Limited Recognition, Michel Morency
Return Receipt Postcard
Express Mail Label No. EF 371228868US
Date of Deposit: May 29, 2001

PCTUS1/REV03

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U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/856940	PCT/FR99/02939	19904-013 NATL

24. The following fees are submitted:.	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**		
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$860.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$130.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	13 - 20 =	0	x $18.00	**$0.00**	
Independent claims	1 - 3 =	0	x $80.00	**$0.00**	
Multiple Dependent Claims **(check if applicable).**			☒	**$270.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,260.00**	
☐ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$0.00**	
SUBTOTAL =				**$1,260.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$1,260.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$1,260.00**	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$1,260.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. ____________ in the amount of ____________ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-0311** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Ivor R. Elrifi
Mintz, Levin, Cohn, Ferris, Glovsky and Popeo, P.C.
One Financial Center
Boston, MA 02111

SIGNATURE

Michel Morency
NAME

Limited Recognition
REGISTRATION NUMBER

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DATE

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PHARMACEUTICAL COMPOSITION COMPRISING AN ANTI-CANCER AGENT AND AT LEAST ONE PEPTIDE

The present invention relates to the use of peptides to carry anti-cancer agents in applications for treating and/or preventing cancer, more particularly in the field of chemoresistance.

A significant problem encountered in the pharmacology of anti-cancer products consists of the intrinsic or acquired resistance of cancerous cells to products. Indeed, it has been observed that cancerous cells from numerous patients suffering from cancer become or are resistant to anti-cancer agents, resulting in the occurrence of new tumoral developments in the patient. Digestive epithelioma, melanoma and kidney cancer are examples of innate chemoresistance. Leukaemia, breast cancer and small-cell bronchial cancer in adults and neuroblastoma in children generally respond well at the beginning of treatment but a large proportion of cases become progressively resistant to treatments.

Primary resistance may be linked to transmembrane transport inhibition and pro-drug activation inhibition mechanisms, modifications in the target enzyme, metabolic processes or repair and inactivation phenomena.

Acquired resistance phenomena are multiple. One of these resistances is multidrug resistance (MDR). MDR is associated with a decrease in the intracellular retention of drugs of differing action mechanisms (Chen et al., 1986, Cell 47, 381-389; Krishan et al., 1997, Cytometry 29, 279-285; Riordan et al., 1985, Nature 316, 817-819).

A membrane-related protein, P-glycoprotein or "P-gp", is the phenotypic expression of the MDR gene. This

protein acts like a dependent power pump transporting cytotoxic drugs outside the cell before they produce their effects. The expression of said protein induces the tumoral cell to resist high concentrations of cytotoxic agents such as doxorubicin, daunomycin, actinomycin D, vinblastin, vincristin, mitomycin C, etoposide, teniposide, etc. The P-gp protein is expressed in normal cells such as those of the gastro-intestinal tract, liver and kidney, where it is thought to eliminate toxins or drugs. It is also thought to be responsible for the low penetration of numerous drugs in the brain.

Therefore, one of the priorities in cancer research consists of finding effective means to circumvent the expression and efficacy of the multidrug resistance phenotype, so as to limit chemotherapy failures. Several studies have been conducted to find agents which inhibit P-gp-related resistance and restore the anti-tumour activity of the cytotoxic product either partially or entirely. Such agents are known as chemosensibilisers or P-gp modulators. Said agents act either directly, by interfering, by competition or steric obstruction, on the fixation sites of the cytotoxic agents, or indirectly, by inhibiting the protein responsible via various mechanisms. A whole range of products are capable of inhibiting multidrug resistance, such as calcium channel inhibitors, phenothiazines, quinidine, anti-malaria agents, anti-oestrogen, cyclosporin. However, the toxicity of these products currently limits their clinical use.

In order to overcome this problem, systems to transport specific anti-cancer agents, particularly doxorubicin, using transferrin (Barabas K. et al., 1992;

The Journal or Biological Chemistry, 267(13): 9347-9442), dextran (Ueda Y. et al., 1989, Chem. Pharm. Bull., 37(6): 1639-41; Sheldon K. et al., 1989, Anticancer Research, 9(3): 637-642), antibodies (Hurwitz E. et al., 1975, Cancer Research, 35: 1175-1181), microspheres (Rogers K. E. et al., 1983, Cancer Research, 43: 2741-2748; Jeanneson P. et al., 1990, Cancer Research, 50, 1231-1236), polymers (Tokoyama M. et al., 1990, Cancer Research, 50: 1693-1700), or protein fragments (Ohkawa K. et al., 1993, British Journal of Cancer, 67: 247-8; Asakura T. et al., 1997, Anticancer Drug, 8(2): 199-203) have been proposed in the prior art. Anti-cancer agent transport means using sequestration in liposomes or nanoparticles (Kruh G. D. and Goldstein L. J., Curr. Opin. Oncol., 5(6): 1029-34; Cuvier C. et al., 1992, Biochemical Pharmacology, 44: 509-517) have also been proposed. However, these systems did not produce satisfactory performances essentially due to their toxicity, a low specificity with respect to cancerous cells, poor storage stability of the finished product and difficult feasibility.

Therefore, the purpose of the present invention is to offer a new, effective and non-toxic means to combat the problem of multidrug resistance. This purpose is achieved through the use of peptides to carry cytotoxic agents to the cancerous target, said peptides also enabling said agents to avoid different resistance mechanisms, particularly the P-gp pump.

Numerous peptides capable of passing through eukaryote membranes very rapidly, such as the following peptides, Protegrin, Antennapedia, Tachyplesin, Transportan, etc. have been described in the prior art.

Of these, some show cytolytic properties. Said peptides, referred to as antibiotic peptides, particularly include Protegrins and Tachyplesins. Protegrins and Tachyplesins are natural antibiotic peptides with a hairpin type structure held by disulphide bridges. Said bridges play an important role in the cytolytic activity observed on human cells.

Depending on their structure, antibiotic peptides can be classified into three main groups:

- Alpha amphipatic helical antibiotic peptides: cecropins and maganins (Maloy, W. L. et al., 1995, BioPolymer 37, 105-122).

- Beta leafed antibiotic peptides joined by disulphide bridges: defensins (Lehrer, R. I. et al., 1991, Cell 64: 229-230; Lehrer, R. I. et al., 1993, Ann. Rev. Immunol. 11: 105-128), protegrins (Kokryakov, V. N. et al., 1993, FEBS 337: 231-236), tachyplesins (Nakamura, T. et al., 1988, J. Biol. Chem. 263: 16709-16713; Miyata, T et al., 1989, J. Biochem. 106: 663-668.

- Destructured chain antibiotic peptides containing numerous bends due to the presence of multiple prolines: bactenecins and PR39 (Frank, R. W. et al., 1991, Eur. J. Biochem. 202, 849-854).

The term protegrins refers to a set of five peptides referred to as PG-1, PG-2, PG-3, PG-4 and PG-5, the sequences of which are given below, closely related and isolated from pig leukocytes (V. N. Kokryakov et el. FEBS lett. 327, 231-236):

PG-1: RGGRLCYCRRRFCVCVGR-NH_2

PG-2: RGGRLCYCRRRFCICV..-NH_2

PG-3: RGGGLCYCRRRFCVCVGR-NH_2

PG-4: RGGRLCYCRGWICFCVGR-NH_2

PG-5: RGGRLCYCRPRFCVCVGR-NH_2

Tachyplesins (Tamura, H. et al., 1993, Chem. Pharm. Bul. Tokyo 41, 978-980), referred to as T1, T2 and T3 and polyphemusins (Muta, T., 1994, CIBA Found. Sym. 186, 160-174), referred to as P1 and P2, the sequences of which are given below, are homologous peptides isolated from the haemolymph of two crabs, *Tachyplesus tridentatus* for the tachyplesins T1, T2 and T3 *and Limulus polyphemus* for the polyphemusins P1 and P2:

P1: RRWCFRVCYRGFCYRKCR-NH_2

P2: RRWCFRVCYKGFCYRKCR-NH_2

Protegrins, tachyplesins and polyphemusins contain a high proportion of basic residue (lysines and arginines) and comprise four cysteines which form two parallel disulphide bridges. These three groups of peptides also show similarities with some defensins, particularly the human defensin NP-1 (Kokryakov, V. N. et al., 1993, Febs Let. 327, 231-236).

In this way, within the scope of this research work, the Applicant discovered that the irreversible reduction of said disulphide bridges makes it possible to obtain linear peptides able to pass through mammalian cell membranes rapidly via a passive mechanism which does not use a membrane receptor. Said linear peptides are non-toxic and free of lytic activity and, as a result, represent a new system for carrying active substances for therapeutic or diagnostic purposes. The research and results related to said linear peptides and their use as vectors of active substances are described in the Applicant's French patent application registered on 12th August 1998 under the No. 97/10297, the content of which is referred to in this disclosure.

Antennapedia group peptides are derived from fruit fly Antennapedia homeodomain transcription factor and are, for example, described in the PCT international patent applications published under Nos. WO91/18981 and WO97/12912. The sequence of said peptides has the specific characteristic of being highly retained in all homeoproteins. Said peptides are composed of three alpha helices and are able to translocate through cell membranes. The smallest fragment of the homeodomain capable of passing through membranes is a peptide comprising 16 amino acids (Prochiantz, 1996, Curr. Opin. In Neurob. 6, 629-634; Derossi et al., 1994, J. Biol. Chem. 269, 10444-10450).

The research work conducted within the scope of the present invention has now enabled the applicant to demonstrate that said linear peptides, i.e. peptides free of disulphide bridges, may be used as a very effective system to carry an anti-cancer substance to a target and enable said substance to pass through the cell membrane to lead it to a cellular compartment such as the cytoplasm or nucleus. Moreover, surprisingly, the Applicant discovered, in addition to their properties as vectors, some of said peptides can be used to prevent the expression of intrinsic or acquired resistance of cancerous cells with respect to said agents, hereafter referred to as chemoresistance, particularly to combat the problem of multidrug resistance (MDR) and enable said agent to avoid the P-gp pump.

Therefore, the invention more particularly relates to a pharmaceutical composition for treating and/or preventing cancer comprising at least one anti-cancer agent, characterised in that said anti-cancer agent is

associated in the composition with at least one peptide capable of carrying said agent into the cancerous cells and preventing the occurrence of chemoresistance to said agent, said peptide complying with one of the following formulas I, II or III:

$X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}-X_{14}-X_{15}-X_{16}$ I

wherein formula I, the residues X_1 to X_{16} are amino acid residues, 6 to 10 of which are hydrophobic amino acids and X_6 is tryptophan,

BXXBXXXXBBBXXXXXXB II

BXXXBXXXBXXXXBBXB III,

wherein formulas II and III:

- the identical or different B groups represent an amino acid residue in which the lateral chain comprises a basic group, and

- the identical or different X groups represent an aliphatic or aromatic amino acid residue,

where the retro form of said formula I, II, III peptides, composed of D and/or L configuration amino acids, or a fragment of said amino acids composed of a sequence of at least 5 and, preferentially, at least 7 successive amino acids of formula I, II or III peptides, provided, of course, that said fragment shows properties as vectors that are non-toxic for the cells.

Formula I peptides are derived from the Antennapedia group. In formula I peptides, the hydrophobic amino acids are alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, tyrosine and methionine, and the other amino acids are:

- non-hydrophobic amino acids which may be non-polar amino acids such as glycine, or polar amino acids such as serine, threonine, cysteine, asparagine, glutamine, or

- acidic amino acids (aspartic or glutamic acid), or
- basic amino acids (lysine, arginine or histidine), or
- a combination of amino acids of these three categories.

Among the formula I peptides, those comprising 6 hydrophobic amino acids and 10 non-hydrophobic amino acids are preferred.

Formula II linear peptides are derived from the Protegrin group and formula III linear peptides are derived by the Tachyplesin group. Among the formula II and III peptides, the following type are preferred, wherein:

- B is selected from arginine, lysine, diaminoacetic acid, diaminobutyric acid, diaminopropionic acid, ornithine, and
- X is selected from glycine, alanine, valine, norleucine, isoleucine, leucine, cysteine, cysteine^{Acm}, penicillamine, methionine, serine, threonine, asparagine, glutamine, phenylalanine, histidine, tryptophan, tyrosine, proline, Abu, carboxylic amino-1-cyclohexane acid, Aib, carboxylic 2-aminotetraline, 4-bromophenyalanine, tert-Leucine, 4-chlorophenylalanine, beta-cyclohexyalanine, 3,4-dichlorophenyalanine, 4-fluorophenyalanine, homoleucine, beta-homoleucine, homophenyalanine, 4-methylphenyalanine, 1-naphthyalanine, 2-naphthyalanine, 4-nitrophenyalanine, 3-nitrotyrosine, norvaline, phenylglycine, 3-pyridyalanine, [2-thienyl]alanine.

In formula I, II or III peptides, B, X and X_1 to X_{16} may be natural amino acids or not, including D configuration amino acids.

Preferred peptides used according to the inventions are selected from those with the following amino acid sequences:

RGGRLSYSRRRFSTSTGR

RGGRLSYSRRRFSVSVGR

KWSFRVSYRGISYRRSR

RRLSYSRRRF

Rqikiwfqnrrmkwkk

CENIKIWLSLRSYLKRR

RGGRLAYLRRRWAVLVGR

where the lower case letters represent D configuration amino acids.

The association of an anti-cancer agent and a peptide defined above in the compositions according to the invention advantageously consists of a link which may be produced by any acceptable linking means given the chemical nature, size and number of associated anti-cancer agents and peptides. It may consist of a covalent, hydrophobic or ionic bond, that may or may not be split in physiological media or inside the cell.

The link may be produced at any of the peptide's sites, in which functional groups such as -OH, -SH, -COOH, NH_2 are naturally present or have been introduced. In this way, an anti-cancer agent can be linked with the peptide at the N-terminal or C-terminal ends or on the peptide's lateral chains.

Similarly, the link may be produced at any site of the active agent, where, for example, functional groups such as -OH, -SH, -COOH, NH_2 are naturally present or have been introduced.

In this way, the invention relates to the use of compounds complying with the following formula IV:

A $(-)_m$ $(B)_n$ IV

where

- A represents a peptide as defined above,
- B represents an anti-cancer agent,
- n is 1 or more, preferably up to 10 and advantageously up to 5,
- $(-)_m$ represents the linker between A and B, where m is 1 or more, preferably up to 10 and advantageously up to 5,

to prepare a drug for treating and/or preventing cancer without inducing chemoresistance.

Formula IV compounds may be prepared chemically or using molecular biology techniques.

For the chemical synthesis, commercial devices enabling the incorporation of non-natural amino acids, such as D enantiomers and residue with lateral chains with different hydrophobicities and sizes than those of their natural counterparts, may be used. During synthesis, it is of course possible to make a wide range of modifications, for example, introduce a lipid such as prenyl or myristyl on the N-terminal, so as to be able to fix the peptide according to the invention and therefore the formula IV compound to a lipidic membrane such as that of a liposome composed of lipids. It is also possible to replace one or more (-CO-NH-) peptide linkers by equivalent structures such as $-CO-N(CH_3)-$, CH_2-CH_2-, $CO-CH_2$, or insert groups such as $-CH_2-$, -NH-, -O-.

It is also possible to obtain formula IV compounds or a protein component thereof from a nucleic acid sequence coding for said compound. The present invention also relates to a nucleic acid molecule comprising or composed of a nucleic sequence coding for an antibiotic

peptide derived linear peptide. More particularly, the invention relates to a nucleic acid molecule comprising at least one sequence coding for a formula IV compound or a protein component thereof. Said nucleic acids may be DNA or RNA and be associated with control sequences and/or inserted in vectors. The vector used is selected according to the host in which it is to be transferred; it may be any vector such as a plasmid. Said nucleic acids and vectors are useful to produce peptides and formula IV compounds or a protein component thereof in a cellular host. The preparation of said vectors and the production or expression of peptides or formula IV compounds in a host may be performed using molecular biology and genetic engineering techniques well known to specialists in the art.

As mentioned above, research work has surprisingly revealed that the peptides defined above are able not only to carry the anti-cancer agent to the cancerous cells, but also to prevent the occurrence of chemoresistance to said agent. As a result, the invention also relates to an inhibition process of the potential ability of an anti-cancer agent to induce chemoresistance in a subject who has received said agent, consisting of associating said agent with at least one formula I, II or III peptide by any suitable means, particularly those described above.

Therefore, the invention also relates to the use of a formula IV compound such as that defined above to prepare an anti-cancer drug also capable of preventing the occurrence of chemoresistance.

According to a preferred embodiment of the above use, said peptide is associated in the drug with the

anti-cancer agent by a linker such as that described above. Preferentially, said linker can be split selectively in the cellular environment. Said drug comprises a pharmaceutically acceptable vehicle compatible with the adopted administration method.

The anti-cancer agents used within the scope of the present invention are all those that are used or can be used in chemotherapy, such as, not exhaustively, doxorubicin, daunomycin, actinomycin D, vinblastin, vincristin, mitomycin C, etoposide, teniposide, taxol, taxoter, methotrexate, etc. Naturally, the invention more particularly relates to anti-cancer agents for which occurrences of chemoresistance have already been observed in exposed subjects.

Within the scope of the present invention, the term anti-cancer agent refers to active substances against P-gp and the gene coding for it, more particularly antibodies or antibody components, nucleic acids or oligonucleotides or ribozymes. Indeed, the present invention also relates to the association of reverse oligonucleotides with the peptides described above to inhibit the expression of P-gp, therefore, useful for treating or preventing cancer while preventing the multidrug resistance phenomenon.

The compositions according to the invention containing formula IV compounds and advantageously a pharmaceutically acceptable vehicle may be administered by different routes including (non-exhaustively), the intravenous, intramuscular, subcutaneous routes, etc.

Other advantages and characteristics of the invention can be seen in the following examples related to the preparation of formula IV compounds where the

anti-cancer agent is doxorubicin and the effect of carrying doxorubicin on its internalisation.

EXPERIMENTAL CONDITIONS

Chemical synthesis

Several peptides were produced and their internalisation was tested in several cell lines. As a general rule, the physico-chemical properties of the peptides were modified and the results obtained demonstrate that, depending on the modification, some peptides show improved penetration over others, such as the peptides of compounds No. 1 to 6 of table I below. It was also observed that some peptides penetrate more rapidly in one cell type than in others, indicating cellular tropism.

Preparation of Doxorubicin-Succ-Peptides

As shown in the synthesis diagram in figure 1, the bonding of doxorubicin on a peptide by means of the succinic link is performed in 3 steps:

Succinic anhydride (1.1 eq, dissolved in DMF) is added to doxorubicin hydrochloride (1 eq), dissolved in dimethylformamide (DMF) in the presence of Diisopropylethylamine (DIEA, 2 eq).

After an incubation period of 20 min at ambient temperature, the doxorubicin hemisuccinate formed in this way is then activated by adding PyBOP (1.1 eq Benzotriazol-1-yl-oxopyrrolidinephosphonium Hexafluorophosphate in DMF) and DIEA (2 eq). This second reaction mixture is incubated for 20 min.

The peptide (1.2 eq in DMF) is then added to the reaction mixture and spontaneously bonds with the doxorubicin hemisuccinate activated during an additional incubation period of 20 min.

The linker is then purified on preparative HPLC (High Pressure Liquid Chromatography) and then freeze-dried.

Each of the steps and the final product are tested using analytical HPLC and mass spectrometry.

Preparation of Doxorubicin-SMP-3MP-Peptide.

As shown in the synthesis diagram in figure 2, the bonding of doxorubicin on a peptide carrying a thiol function is performed in 2 steps:

N-hydroxy-Succinimidyl-Maleimido-Propionate (SMP, 1 eq, dissolved in DMF) is added to doxorubicin hydrochloride (1 eq), dissolved in dimethylformamide (DMF) in the presence of Diisopropylethylamine (DIEA, 2 eq).

The peptide carrying a thiol function (1.2 eq in DMF) is then added to the reaction mixture and spontaneously bonds onto the doxorubicin maleimidopropionate during an additional incubation period of 20 min.

The linker is then purified on preparative HPLC (High Pressure Liquid Chromatography) and then freeze-dried.

Each of the steps and the final product are tested using analytical HPLC and mass spectrometry.

Tested products

The tested products are given in table I below.

Table I

Compound	
1	doxo-CO-(CH2)$_2$-CO- RGGRLSYSRRRFSTSTGR
2	doxo-CO-(CH2)$_2$-CO- RGGRLSYSRRRFSVSVGR
3	doxo-CO-(CH2)$_2$-CO- KWSFRVSYRGISYRRSR

4	doxo-CO-(CH2)$_2$-CO- RRLSYSRRRF
5	doxo-SMP-3MP-rqikiwfqnrrmkwkk
6	doxo-SMP-CENIKIWLSLRSYLKRR
7	doxo-CO-(CH2)$_2$-CO-RGGRLAYLRRRWAVLVGR

doxo = doxorubicin

CO-(CH2)$_2$-CO = Succinate linker

SMP-3MP = SuccinimydylMaleimido-Propionate-3-MercaptoPropionate linker

Cell culture

The susceptible K562 chronic myeloid leukaemia cells, resistant K562/ADR cells and HL60/R10 promyeloid leukaemia cells are of human origin and were obtained commercially from the ATCC. The cells are inoculated at a rate of approximately 10^4 cells per well, 24 hours before adding the products. The cells are kept in culture at 37°C in an atmosphere at 95% humidity and 5% CO_2 in an OptiMem medium.

Internalisation

The susceptible K562 cells and resistant K562/ADR cells are incubated either with free doxorubicin or with doxorubicin in a vector at a concentration of 3 µM. After 30 minutes of incubation at 37°C, the cells are washed three times with PBS. The internalisation of the products is then analysed by flow cytometry. The samples are analysed with a flow cytometer equipped with a 15 mA argon laser. The fluorescence emitted is recorded on a logarithmic scale at (575 NM) nm after an excitation at (488) nm. The fluorescence is measured on 10,000 cells selected according to the size SS and granulosity FS parameters. The results are expressed as a percentage of positive cells of the fluorescence intensity logarithm.

The results are analysed using the software program Cell Quest.

Cytotoxicity

The susceptible K562 cells and resistant K562/ADR cells are incubated either with free doxorubicin or with doxorubicin in a vector at increasing concentrations after 48 hours in culture in the presence of the products. At the end of the culture period, MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) is added into the wells and the culture plates are then incubated for 4 hours in the incubator. The resulting crystalline formazan deposit is then dissolved by adding 200 µl of DMF/SDS. The optical density (OD) is measured at 550 nm (reference 630 nm) using a microplate reader.

The graphic representation of the OD percentages of the wells treated as a function of the concentration of products is used to determine the IC_{50} which corresponds to the concentration of product that inhibits 50% of growth.

The IC_{50} values are used to quantify the resistance factor (F. Res) of the resistant lines using the following ratio:

$$\text{F. Res} = \frac{\text{Cytotoxic } IC_{50} \text{ for resistant line}}{\text{Cytotoxic } IC_{50} \text{ for susceptible parental line}}$$

The reversion factor (F. Rev) corresponds to the effect of a modulator (the vector) on the susceptibility of the cells to the anti-tumour agents according to the following ratio:

$$\text{F. Rev} = \frac{\text{Cytotoxic } IC_{50} \text{ alone}}{\text{Cytotoxic } IC_{50} \text{ in vector}}$$

RESULTS

Internalisation

The susceptible K562 cells and resistant K562/ADR cells are incubated either with free doxorubicin or with doxorubicin in the vector. After 30 min of incubation, the internalisation of the products is measured by flow cytometry. Figure 3 in the appendix shows that, in the resistant K562/ADR cells, only 5.86% of the cells are positive, while with the vector (e.g. in the form of compound No. 5 in table I), 98% of the cells are positive, indicating a marked improvement in penetration.

Cytotoxicity

The susceptibility of the cells to anti-tumour agents was measured using the MTT test under the experimental conditions defined above wherein the relationship between the optical density and the number of viable cells is linear.

The activity of doxorubicin in the susceptible cells (K562) and in the resistant cells K562/ADR was first of all observed. The cells are incubated with increasing concentrations of doxorubicin and after 24 hours of incubation, the survival of the cells is measured using the MTT test. As shown in Figure 4, the K562/ADR cells are indeed resistant to doxorubicin. For example, at a concentration of 8 μM, only 15% of the susceptible cells survive while 100% of the resistant cells survive.

The cytotoxicity of doxorubicin in the vector was then analysed. Table II below represents the concentration of product inducing 50% growth inhibition IC_{50} determined on the susceptible line K562 and the resistant line K562/ADR.

Table II

Lines	K562	K562/ADR	F. Res	F. Rev
Free Doxo	0.9 µM	25 µM	28	
Compound 1	15 µM	5-10 µM	0.7	2.5
Free Doxo	0.15 µM	15.5 µM	100	
Compound 2	3.6 µM	3.2 µM	0.9	5
Free Doxo	0.45 µM	65 µM	144	
Compound 3	2 µM	1.5 µM	0.8	43
Free Doxo	ND	55 µM		
Compound 4	20 µM	20 µM	1	2.8
Free Doxo	0.4 µM	70 µM	175	
Compound 5	1.5 µM	2 µM	1.3	35
Free Doxo	0.3 µM	70 µM	233	
Compound 6	5 µM	5.5 µM	1.1	12.8
Free Doxo	0.1 µM	> 78 µM	> 780	
Compound 7	3.5 µM	3 µM	0.85	> 26

These results show that the K562/ADR cells are indeed resistant to doxorubicin alone. Carrying the doxorubicin in a peptide-vector makes it possible to circumvent the problem of multidrug resistance. For example, in one case, the IC_{50} of free doxorubicin in the resistant cells is 70 µM, while with the vector (compound 5), the IC_{50} is only 2 µM.

These results also show that the products in vectors are not excreted by the P-gp pump since the same IC_{50} is obtained in the susceptible cells and resistant cells and the resistance factor is almost 1.

To be certain that the cytotoxicity observed for doxorubicin in vectors is not due to the peptide alone, an experiment was conducted, comparing the activity of doxorubicin in the vector (compound 2) with doxorubicin

added to, but not linked with, the peptide. Figure 5 appended shows that the IC_{50} of doxorubicin in the vector (compound 2) is 19 µM while that of doxorubicin added to the vector is approximately 50 µM, thus demonstrating the need to introduce a vector to reduce cell resistance.

The same type of experiment was conducted on another cell line resistant to doxorubicin HL60/R10. The cytotoxicity results in the HL60 cells with compound No. 2 are given in table III below.

Table III

	HL60/R10	F. Rev
Free doxo	40 µm	
Compound No. 2	25 µm	1.6

CLAIMS

1) Pharmaceutical composition for treating and/or preventing cancer comprising at least one anti-cancer agent, characterised in that said anti-cancer agent is associated in the composition with at least one peptide capable of carrying said agent into the cancerous cells and preventing the occurrence of chemoresistance to said agent, said peptide complying with one of the following formulas (I), (II) or (III):

$X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}-X_{14}-X_{15}-X_{16}$ (I)

wherein formula (I), the residues X_1 to X_{16} are amino acid residues, 6 to 10 of which are hydrophobic amino acids and X_6 is tryptophan,

BXXBXXXXBBBXXXXXXB (II)

BXXXBXXXBXXXXBBXB (III),

wherein formulas (II) and (III):

- the identical or different B groups represent an amino acid residue in which the lateral chain comprises a basic group, and

- the identical or different X groups represent an aliphatic or aromatic amino acid residue,

where the retro form of said formula (I), (II), (III) peptides, composed of D and/or L configuration amino acids, or a fragment of said amino acids composed of a sequence of at least 5 and, preferentially, at least 7 successive amino acids of formula (I), (II) or (III) peptides.

2) Composition according to claim 1, characterised in that in the formula (I) peptide, the hydrophobic amino acids are alanine, valine, leucine, isoleucine, proline,

phenylalanine, tryptophan, tyrosine and methionine, and the other amino acids are:

- non-hydrophobic amino acids which may be non-polar amino acids such as glycine, or polar amino acids such as serine, threonine, cysteine, asparagine, glutamine, or
- acidic amino acids (aspartic or glutamic acid), or
- basic amino acids (lysine, arginine or histidine), or
- a combination of amino acids of these three categories.

3) Composition according to any of claims 1 or 2, characterised in that the formula (I) peptide comprises 6 hydrophobic amino acids and 10 non-hydrophobic amino acids.

4) Composition according to claim 1, characterised in that in the formula (II) or (III) peptides:

- B is selected from arginine, lysine, diaminoacetic acid, diaminobutyric acid, diaminopropionic acid, ornithine, and
- X is selected from glycine, alanine, valine, norleucine, isoleucine, leucine, cysteine, cysteine^{Acm}, penicillamine, methionine, serine, threonine, asparagine, glutamine, phenylalanine, histidine, tryptophan, tyrosine, proline, Abu, carboxylic amino-1-cyclohexane acid, Aib, carboxylic 2-aminotetraline, 4-bromophenylanine, tert-Leucine, 4-chlorophenylalanine, beta-cyclohexyalanine, 3,4-dichlorophenyalanine, 4-fluorophenyalanine, homoleucine, beta-homoleucine, homophenyalanine, 4-methylphenyalanine, 1-naphthyalanine, 2-naphthyalanine, 4-nitrophenyalanine, 3-nitrotyrosine, norvaline, phenylglycine, 3-pyridyalanine, [2-thienyl]alanine.

5) Use of a compound complying with the following formula (IV):

A $(-)_m$ $(B)_n$ (IV)

where

- A represents a peptide as defined above,
- B represents an anti-cancer agent,
- n is 1 or more, preferably up to 10 and advantageously up to 5,
- $(-)_m$ represents the linker between A and B, where m is 1 or more, preferably up to 10 and advantageously up to 5,

to prepare a drug for treating and/or preventing cancer without inducing chemoresistance.

6) Use according to claim 5, characterised in that, in formula (IV) the linker $(-)_m$ between A and B is a covalent, hydrophobic or ionic bond, that may or may not be split in physiological media or inside the cell, or a combination of said bonds.

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(71) Déposant *(pour tous les Etats désignés sauf US)*: SYNT:EM (S.A.) [FR/FR]; Parc Scientifique Georges Besse, F-30000 Nîmes (FR).

(72) Inventeurs; et
(75) Inventeurs/Déposants *(US seulement)*: TEMSAMANI, Jamal [FR/FR]; Résidence de l'Empereur, Bâtiment B, 26, chemin des Carrières, F-30900 Nîmes (FR). KACZOREK, Michel [FR/FR]; 81, boulevard de la Lironde, F-34980 Montferrier sur Lez (FR). COLIN DE VERDIERE, Annik [FR/FR]; 113, allée des Pins, Résidence Claude Monet, F-30000 Nîmes (FR).

(74) Mandataires: BREESE, Pierre etc.; Breese-Majerowicz, 3, avenue de l'Opéra, F-75001 Paris (FR).

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(54) Title: PHARMACEUTICAL COMPOSITION COMPRISING AN ANTI-CANCER AGENT AND AT LEAST A PEPTIDE

(54) Titre: COMPOSITION PHARMACEUTIQUE COMPRENANT UN AGENT ANTI-CANCEREUX ET AU MOINS UN PEPTIDE

(57) Abstract

The invention concerns a pharmaceutical composition for treating and/or preventing cancer comprising at least an anti-cancer agent, characterised in that said anti-cancer agent is associated in the composition with at least a peptide capable of carrying said agent into the cancer cells and prevent the occurrence of chemoresistance to said agent.

(57) Abrégé

La présente invention concerne une composition pharmaceutique destinée au traitement et/ou à la prévention des cancers comprenant au moins un agent anti-cancéreux, caractérisée en ce que ledit agent anti-cancéreux est associé dans la composition avec au moins un peptide capable de transporter ledit agent dans les cellules cancéreuses et d'empêcher l'apparition d'une chimiorésistance vis-à-vis dudit agent.

Figure 1: Preparation of doxorubicin-Succ-peptides

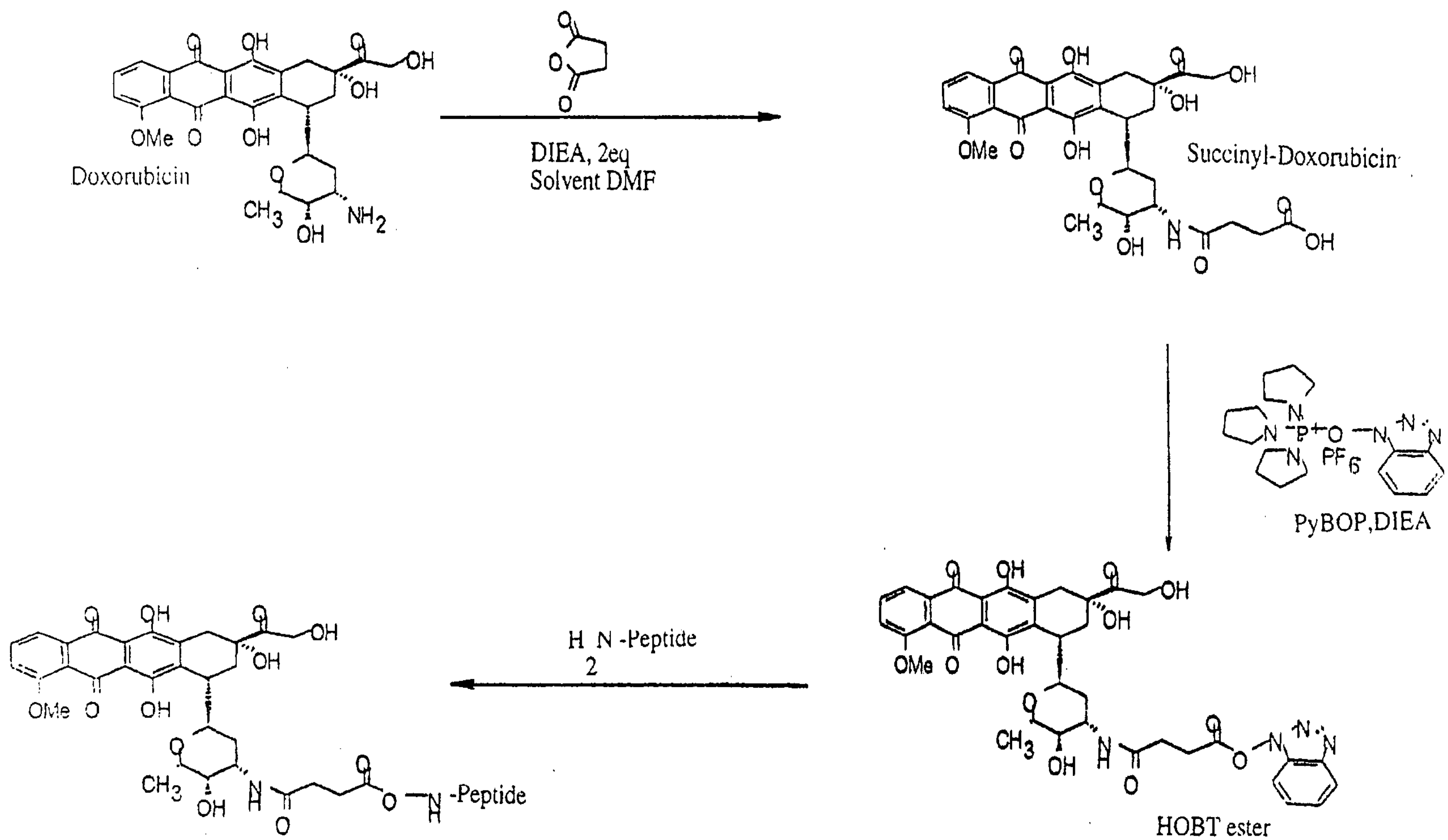


Figure 2: Preparation of doxorubicin-SMP-3MP-peptides

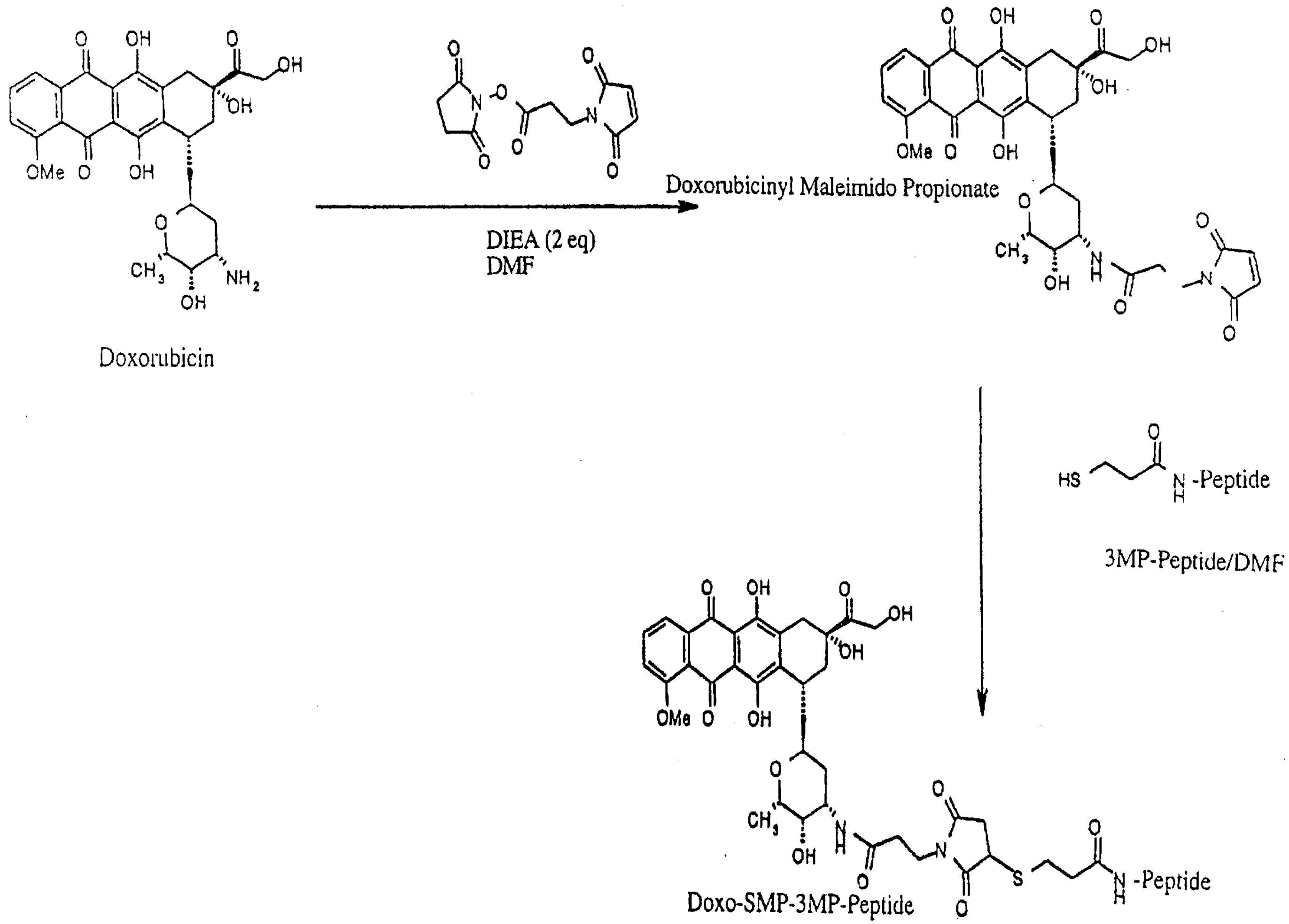


Fig. 3

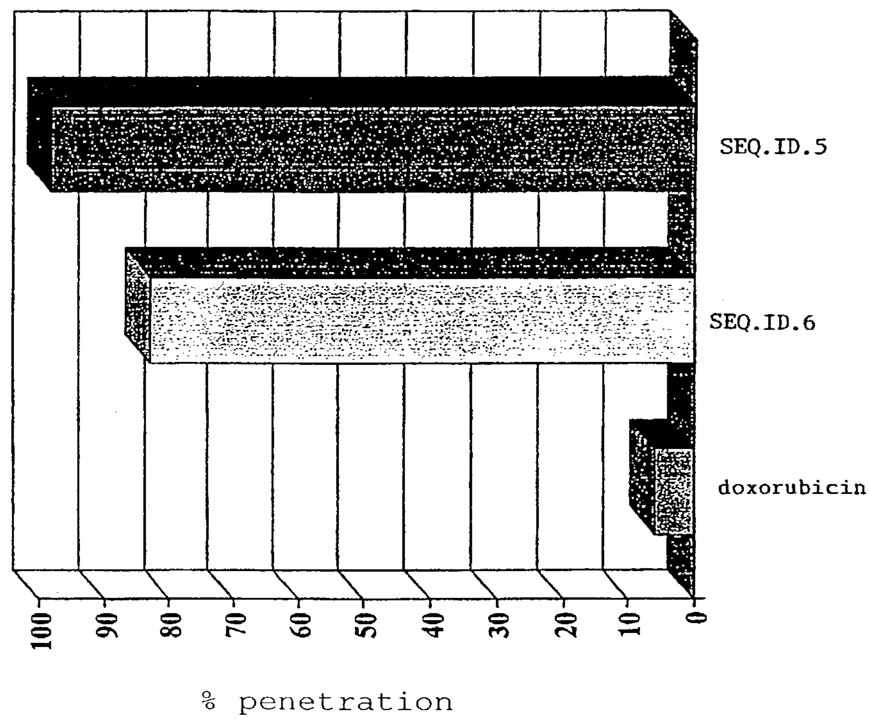

SEQ.ID.5
SEQ.ID.6
doxorubicin
100
90
80
70
60
50
40
30
20
10
0
% penetration

Fig. 4

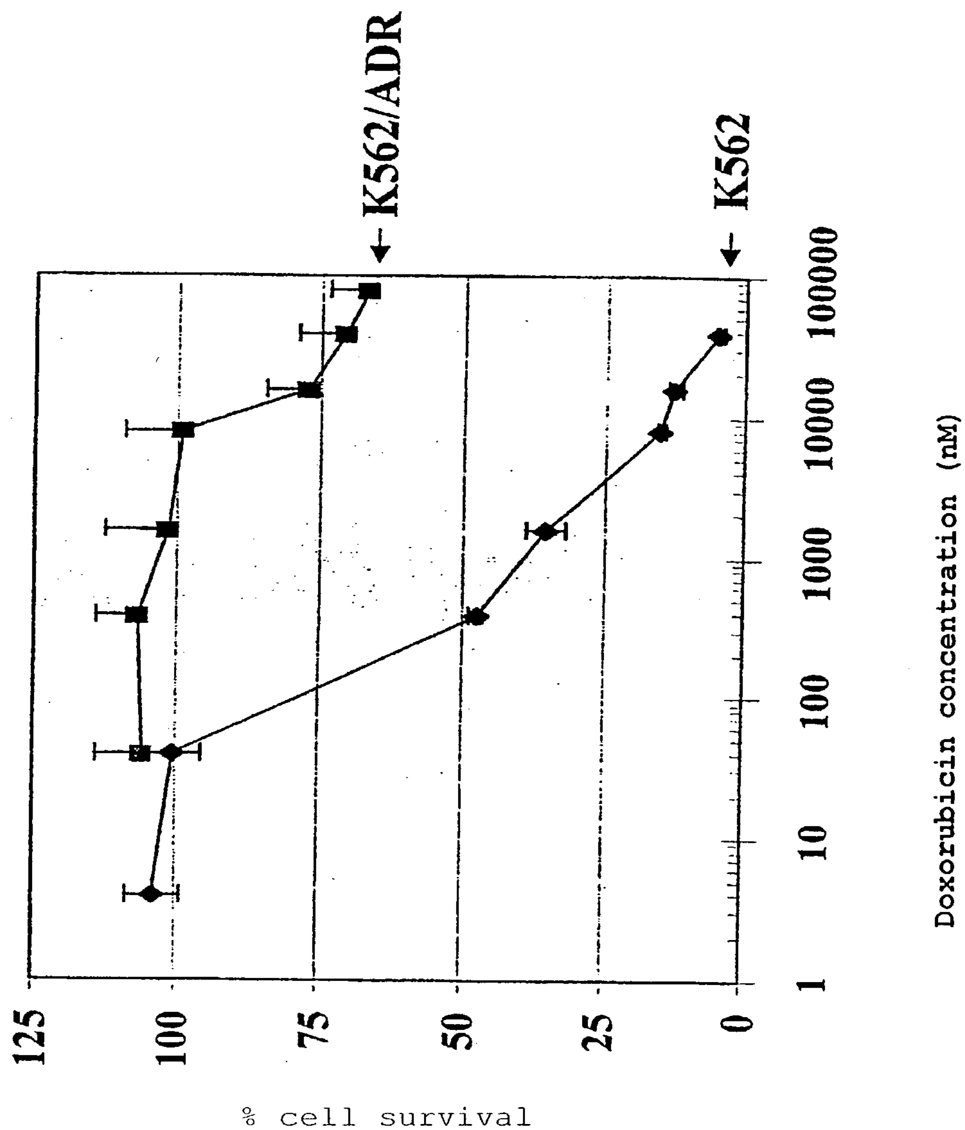
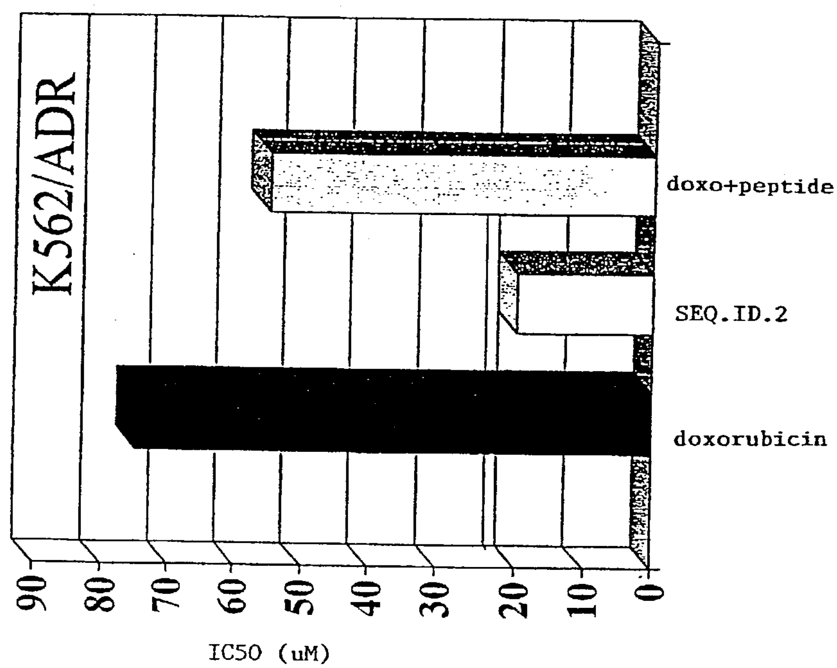

K562/ADR
K562
125
100
75
50
25
0
1
10
100
1000
10000
100000
% cell survival
Doxorubicin concentration (nM)

Fig. 5


K562/ADR
doxo+peptide
SEQ.ID.2
doxorubicin
90
80
70
60
50
40
30
20
10
0
IC50 (uM)

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Date of Deposit: December 6, 2001

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Attorney Docket No. 19904-013 NATL.
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As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a utility patent is sought on the invention entitled:

PHARMACEUTICAL COMPOSITION COMPRISING AN ANTI-CANCER AGENT AND AT LEAST A PEPTIDE

☒ was filed on November 26, 1999 as a PCT application designating the United States, and was assigned PCT/FR99/02939. A United States national phase application was filed on May 30, 2001 and given U.S. Serial No. 09/856,940.

☐ is attached hereto.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

☐ I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application designating at least one country other than the United States listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Appln. Number	Country *(if PCT, so indicate)*	Filing Date *(dd/mm/yy)*	Priority Claimed	
			Yes	No
			☐	☐
			☐	☐
			☐	☐
			☐	☐
			☐	☐
			☐	☐

☒ I hereby claim the benefit under Title 35, United States Code, § 119(e) or §120 of any United States application(s), or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

Application No. *(U.S.S.N.)*	**Filing Date** *(dd/mm/yy)*	**Status** *(Patented, Pending, Abandoned)*

PCT International Applications designating the United States:

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PCT/FR99/02939	26 Nov. 1999	pending

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

37

Attorney or Agent	**Registration No.**	**Attorney or Agent**	**Registration No.**
Kevin N. Ainsworth	39,586	Kris Kalidindi	41,461
Ingrid A. Beattie	42,306	Christina V. Karnakis	45,899
William Belanger	40,509	Kristin E. Konzak	44,848
Naomi Biswas	38,384	Cynthia Kozakiewicz	42,764
Bradford C. Blaise	47,429	Barry J. Marenberg	40,715
Duane Blake	47,279	William A. Marino	44,219
Yong Choi	43,324	A. Jason Mirabito	28,161
David F. Crosby	36,400	Michel Morency	Limited Recognition
Christopher J. Cuneo	42,450	Carol H. Peters	45,010
Brett N. Dorny	35,860	David W. Poirier	43,007
Marianne Downing	42,870	Michael Renaud	44,299
Ivor R. Elrifi	39,529	Brian Rosenbloom	41,276
Heidi A. Erlacher	45,409	Robert J. Sayre	42,124
James G. Gatto	32,694	Thomas M. Sullivan	39,392
Richard Gervase	P-46,725	Janine M. Susan	46,119
Matthew J. Golden	35,161	Howard Susser	33,556
John A. Harre	37,345	Raphael A. Valencia	43,216
Brian P. Hopkins	42,669	Shelby J. Walker	45,192
Shane Hunter	41,858		
David E. Johnson	41,874		

all of MINTZ, LEVIN, COHN, FERRIS, GLOVSKY AND POPEO PC, One Financial Center, Boston, Massachusetts 02111, as Applicant's attorneys with full power of substitution and revocation to take any and all action necessary with regard to the above-identified patent.

Address all telephone calls to Ivor R. Elrifi at telephone number 617/348-1747.
Address all correspondence to:

Ivor R. Elrifi
Mintz, Levin, Cohn, Ferris, Glovsky and Popeo, P.C.
One Financial Center
Boston, Massachusetts 02111

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issued thereon.

1-00

Inventor's Signature — Date: August 27, 2001.

Full Name of Inventor: Jamal TEMSAMANI
Citizenship: France
Residence: Résidence de l'Empereur, Bâtiment B
Post Office Address: 26, chemin des Carrières, F-30900 Nîmes, FRANCE FRX

2-00

Inventor's Signature — Date: 27 August 2001.

Full Name of Inventor: Michel KACZOREK
Citizenship: France
Residence: 81, boulevard de la Lironde,
Post Office Address: F-34980 Montferrier sur Lez, FRANCE FRX

Inventor's Signature — Date

Full Name of Inventor: Annik COLIN DE VERDIERE
Citizenship: France
Residence: 113, allée des Pins,
Post Office Address: Résidence Claude Monet, F-3000 Nîmes, FRANCE

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